Cloning, Mapping, and Expression of the Fumarase Gene of *Escherichia coli* K-12

JOHN R. GUEST* AND RUTH E. ROBERTS

Department of Microbiology, Sheffield University, Sheffield S10 2TN, United Kingdom

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Two classes of fumarase-transducing phages, \(\lambda fumA\) and \(\lambda fumB\), were isolated from populations of recombinant phages containing HindIII fragments of *Escherichia coli* DNA; they were isolated by virtue of their ability to complement the metabolic lesion of a fumarase-negative mutant. The strongly complementing \(\lambda fumA\) phages contained a 6.2-kilobase HindIII fragment encoding: the *fumA* gene, located at 35.5 min in the *E. coli* linkage map and expressing the major fumarase activity; the mannosephosphate isomerase gene, *manA*; and an unidentified gene, *g48*. The three genes were located relative to the restriction map of the cloned fragment and the genetic linkage map (terC-*g48-fumA-manA-uidA0R*), their transcription polarities were defined as anticlockwise in the chromosome, and the molecular weights of the corresponding gene products were established: *fumA*, 61,500; *manA*, 42,000; *g48*, 48,000. Organisms containing the *fumA* gene sub-cloned in multicopy plasmids overproduced fumarase up to 50-fold. The weakly complementing class of transducing phages, \(\lambda fumB\), contained several genes in an 8.2-kilobase HindIII fragment, including one (*fumB*) that determines a minor fumarase activity. Complementation by *fumB* was only observed in high-copy situations such as transduction plaques and in strains containing a multicopy plasmid in which 40% of normal fumarase activity was detected. The basis for the complementation by *fumB* was not defined.

Fumarase hydratase or fumarase (EC 4.2.1.2) catalyzes the interconversion of fumarate and malate in the tricarboxylic acid cycle. It is widely distributed in animals, plants, and microorganisms, but the mammalian enzymes have been studied most; the fumarase of *Escherichia coli* has received very little attention.

Molecular cloning of the tricarboxylic acid cycle genes of *E. coli* has so far relied on nutritional complementation of the corresponding mutants for detecting either phage- or plasmid-cloned genes. This approach depends on the availability of mutants with lesions in the metabolic cycle, and it has been successfully applied to the genes for citrate synthase (*gltA*), succinate dehydrogenase (*sdh*), 2-oxoglutarate dehydrogenase (*sucA*), dihydrolipoamide succinyltransferase (*sucB*), and lipoamide dehydrogenase (*lpd*) (11, 13, 24). In the case of fumarase, this limitation has been overcome by the recent isolation of a fumarase-negative mutant by Eric J. Hansen and Elliot Juni (personal communication). The mutant possesses two features which provide convenient selections for gene cloning by nutritional complementation: the inability to use fumarate as growth substrate, and very poor growth on a weak casein digest (peptone) medium. Accordingly, pools of artificially constructed \(\lambda\) phages containing HindIII and EcoRI fragments of *E. coli* DNA were screened for the presence of fumarase-transducing phages (\(\lambda\) *fum*). This paper describes the isolation of two classes of phages, \(\lambda\) *fumA* and \(\lambda\) *fumB*, both containing HindIII fragments (6.2 and 8.2 kilobases [kb], respectively) but differing in their ability to complement the fumarase defect of the *fumA* mutant. Restriction maps of the corresponding segments of bacterial DNA were constructed, and some of their constituent genes were characterized by size of gene product and by transcription polarity. The *fumA* gene encoding the major fumarase was located near the chromosome replication terminus (terC) and very close to the *manA* gene at 35.5 min. The fumarase and mannosephosphate isomerase gene products were identified as polypeptides of \(M_r\) 61,500 and 42,000, respectively. In strains containing *fumA* plasmids, the fumarase activities were amplified up to 50-fold.

MATERIALS AND METHODS

Bacteria. The bacterial strains are listed in Table 1. The fumarase mutant, 1535, was kindly provided by E. Juni, and the fumarase mutation has been designated *fumA*. It was isolated after nitrosoguanidine mutagenesis of strain NK-1, a parental strain that grows...
TABLE 1. Strains of E. coli K-12

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype*</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td>NK-1</td>
<td>spoT</td>
<td>E. Juni</td>
</tr>
<tr>
<td>15</td>
<td>spoT fumA1</td>
<td>E. Juni</td>
</tr>
<tr>
<td>JRG1331</td>
<td>spoT fumA1 gyrA</td>
<td>Na1 derivative of 1535</td>
</tr>
<tr>
<td>C600</td>
<td>thr-1 leuB6 thi-1 supE44 tonA21 lacY recA λ'</td>
<td>N. E. Murray</td>
</tr>
<tr>
<td>PM191/A</td>
<td>thr-1 leuB6 thi-1 supE44 tonA21 lacY recA λ'</td>
<td>λ' derivative of PM191 from P. Meacock</td>
</tr>
<tr>
<td>S159</td>
<td>gal uvrA rpsL sup*</td>
<td>N. E. Murray</td>
</tr>
<tr>
<td>AB2480</td>
<td>pro thi uvrA6 recA8</td>
<td>P. J. Emmerson</td>
</tr>
</tbody>
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* All strains are F− and λ− unless otherwise indicated.

vigorously on fumarate. Strain C600 was used for routine phage propagation and assay, and strain PM191/A was used as recipient in transformation. A collection of donor strains, including Hfr (H, KL96, KL99) and F− (F123, F129, F150, F500, F506) derivatives, were kindly provided by B. J. Bachmann for conjugation studies (1).

**Bacteriophages.** The phages used were λNM540 (ΔσΔλA-1-2 shnλ3 att' imm21 nin5 shnλ6') and λNM761 (ΔσΔλA-1-2 shnλ3 (att-red) imm21 nin5 shnλ6') as HindIII insertion and replacement vectors (11), and λNM781 (ΔσΔλA-1-3 c1857 nin5; Murray et al., 20) and λNM816 (Wilson and Murray, 26) as EcoRI replacement vectors. Pools of recombinant phages, obtained by inserting the corresponding fragments of E. coli DNA (strain CR63, supD λ'), were kindly provided by N. E. Murray (λNM540, λNM781) or constructed (λNM761, λNM816). Other phages, λ b2 c imm', λ b2c imm21, and λ vir, were used in lysogen selection and for routine testing of immunity and sensitivity.

**Media.** The rich medium for subculturing and phage propagation was L broth or LB broth if used with tricarboxylic acid cycle mutants (11). A peptone medium (10) was used for phage assays and for selective purposes. The minimal medium E of Vogel and Bonner (26) was used with glucose (11 mM), mannose (11 mM), and potassium salts of fumaric, acetic, and DL-malic acids (40 mM) as carbon sources and was supplemented as required with arginine (30 μg/ml) and thiamine (5 μg/ml). Media were solidified with agar (Difco Laboratories) at concentrations of 15 or 10 g/liter for plates and 6.5 g/liter for top layers. Ampicillin (50 μg/ml), tetracycline (25 μg/ml), and nalidixic acid (10 μg/ml) were added to media for selecting and testing conjugants and transformants.

**Genetic methods.** Conjugations were performed by cross-streak tests on solid media by the method of Lambden and Guest (15) or by interrupted mating and time-of-entry mapping (19) with fumarate minimal medium. Transduction was performed as described previously (8) with peptone (for Fum') and mannose minimal (for Man+) selective media. Transformation with plasmid DNA was by the method of Lederberg and Cohen (16) with L agar plus antibiotics or appropriate minimal media for selection purposes.

**Isolation and manipulation of DNA.** The methods for isolating phage and plasmid DNA have been described previously (24), as have the methods for restriction endonuclease digestion, agarose gel electrophoresis, and DNA ligation (24). Small-scale plasmid preparations (3) were used routinely for plasmid screening and transformation.

**Cell extracts and enzymology.** Cultures for enzymology were grown from exponential LB broth inocula (0.05 ml) in glucose minimal medium (250 ml in a 2-liter conical flask) with shaking at 37°C and were harvested in early stationary phase. Twice-washed suspensions of 0.2 g (wt weight) of cells per ml of 0.04 M phosphate buffer (pH 7.8) were disrupted for two periods of 2 min each with an ultrasonic cell disintegrator (M.S.E., 150 W) at 0°C. Debris was removed by centrifuging at 15,000 × g for 20 min, and the supernatants were spun at 100,000 × g for 60 min to provide the high-speed supernatant extracts used for the enzyme and protein assays (17). Fumarase (EC 4.2.1.2) was assayed spectrophotometrically at 240 nm and 25°C by two methods: (i) by following the disappearance of fumarate by the method described by Reeves et al. (22) with an initial fumarate concentration of 0.5 mM, and (ii) by measuring the conversion of L-malate to fumarate by the method of Hill and Bradshaw (14). Aconitase (EC 4.2.1.3) was assayed similarly by following the disappearance of aconitate by the method of Fansler and Lowenstein (9). Specific activities are expressed as micromoles of substrate transformed or product formed per milligram of protein per hour at 25°C in the high-speed supernatant extracts.

**Analysis of polypeptides formed in UV-irradiated hosts.** The polypeptides synthesized after infecting UV-irradiated malate-grown aerobic cultures of strains S159 and S159 (λ imm21) with λ fum transducing phages were labeled with L-[1-14C]methionine as described previously (12). Polypeptides expressed from plasmid-coded genes were labeled by the maxicell procedure of Sancar et al. (23) by using glucose-grown cultures of strain AB2480 transformed with the relevant plasmids. Radioactive polypeptides were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with 10% (wt/vol) acrylamide and with appropriate molecular weight standards in the range 12,500 to 116,000 to determine molecular weights as described previously (12).

**Materials.** Restriction enzymes were purchased from Boehringer Co., Ltd., Bethesda Research Laboratories, Inc., and New England Biolabs, Inc. T4 DNA ligase was from Bethesda Research Laboratories, Inc., and L-[1-14C]methionine (80 to 125 μCi/ml) was purchased from the Radiochemical Centre, Amer sham, U.K.
RESULTS

Location of the fumarase gene (fumA). A fumarase-negative mutant of E. coli, strain 1535, was kindly provided by E. Juni. It was selected by virtue of its ability to use L-malate but not fumarate as the sole source of carbon and energy. Further tests showed that the mutant cannot use acetate as a substrate and that growth on a casein digest (peptone medium) is severely impaired, whereas growth on unsupplemented glycerol and glucose media is only slightly impaired. This nutritional phenotype is consistent with a metabolic lesion in the tricarboxylic acid cycle.

The fumA locus was mapped by conjugation with strain JRG1331, a nitrosoguanidine-induced nalidixic acid-resistant derivative of 1535, as recipient and a variety of Hfr and F' donors. Fum+ conjugants were selected on fumarate minimal medium, and the donors were counter-selected with nalidixic acid (10 μg/ml) and/or nutritionally for auxotrophs. In cross-streak conjugations, positive results were obtained with three Hfr donors (HfrH, KL99, and KL96), indicating that the fumA marker is located in the 22- to 45-min sector of the linkage map (1). Further studies involving time-of-entry mapping with HfrH and cross-streak conjugations with appropriate F' strains placed the fumA marker in the 35- to 37-min region not far from the replication terminus (Fig. 1).

Isolation and properties of λ fum transducing phages. Pools of recombinant phages containing HindIII and EcoRI fragments of E. coli DNA were screened for their ability to complement the fumA mutation. By means of the weakly selective peptone medium that has been employed successfully with other tricarboxylic acid cycle mutants (10, 24), transduction plaques were detected with the HindIII pools but not with the EcoRI pools. Transducing phage derivatives (λ fum) of both HindIII vectors (λNM540 and λNM761) were purified by selective and nonselective single-plaque isolation, and two distinct classes were detected. They were distinguished on the basis of their ability to form either densely turbid-centered plaques within 2 days (λ fumA) or weaker plaques developing over 3 to 4 days (λ fumB). The strong transducers (λ fumA) are represented by λG134 and λG135 (λNM540 derivatives) and λG137 and λG138 (λNM761 derivatives), whereas the weak transducers (λ fumB) are represented by λG139 and λG140 (λNM540 derivatives) and λG141 (λNM761 derivative).

Twelve independently isolated λ fum transducing phages were compared with each other and their vectors by digesting the phage DNAs with HindIII and EcoRI, separately and in combination, and analyzing the products by agarose gel electrophoresis. Seven different restriction patterns, corresponding to the representative phages described above, were identified (Fig. 2). The λ fumA phages were characterized by the presence of a 6.2-kb HindIII fragment containing a single EcoRI target which generates double-digestion products of 2.7 and 3.5 kb. Phage

FIG. 1. Scale map showing origins and polarities of Hfr and F' donor strains used in conjugation studies to locate the fumA gene. The approximate position of the fumA gene (35 to 37 min) is denoted by the + symbol associated with the donor strains that generate Fum+ conjugants. Distances are indicated in minutes; strains and symbols are from Bachmann and Low (1).

FIG. 2. Restriction patterns of the λ fum transducing phages and their vectors. The relative positions of the HindIII (▼) and EcoRI (▲) targets and the sizes of bacterial HindIII and HindIII plus EcoRI fragments (in kilobases) are indicated. Phage DNA is represented by the single lines (not to scale) with arrowheads marking the left arms and ▪ denoting the prophage attachment sites, present in phage λNM540 and derivatives but deleted from phage λNM761 and derivatives. The segments of cloned bacterial DNA are denoted by open bars, drawn to scale.
The λ fumA phages were tested for their ability to transduce the gene for mannosephosphate isomerase (EC 5.3.1.8, manA), which had previously been located at 35.5 min in the E. coli linkage map (1, 18), and should be close to the fumA gene. The λ fumB phages exhibited high frequencies of Man+ transduction (0.1 to 0.5 per PFU) with strain GMS407 as the recipient, but the λ fumB phages were inactive. It was also found that lysogenic derivatives of strain GMS407, isolated nonselectively, always gained the Man+ phenotype with λ fumA prophages but never with λ fumB prophages. This indicates that the segment of bacterial DNA cloned in the λ fumA phage contains a functional manA gene that is expressed from its own promoter.

Properties of λ fum lysogens. Lysogenic derivatives of the fumarase-negative mutant (1535) and its parent (NK-1) containing λ fum and vector prophages were isolated and examined nutritionally and enzymologically.

(i) Nutritional characteristics. Qualitative growth tests with plates of minimal media containing glucose, malate, fumarate, and acetate showed that lysogeny with λG134 and λG135 (λ fumA) restored growth on fumarate and acetate to the fumarase mutant. In contrast, lysogeny with λG139 and λG140 (λ fumB) and the vector phage had no detectable effect on the mutant phenotype. The parental strain (NK-1) grew well on all substrates, and no differences were detected with lysogenic derivatives.

(ii) Enzymological characteristics. Fumarase was assayed in both the forward and reverse directions by following the disappearance of fumarate and the conversion of malate to fumarate; the specific activities are recorded in Table 2. Lysogeny with λ fumA restored the fumarase activities of strain 1535 to levels slightly higher than those of the parental strain. The presence of λ fumA prophages also resulted in two- to threefold increases in the fumarase activities of the parental strain, presumably because of a gene dosage effect. This confirms that the λ fumA phages contain an active form of the fumarase gene that is inactive in the fumA strain, 1535. Because the relevant vector promoters are repressed in the prophage state, it is very likely that the fumA gene is expressed from its own promoter. The enzyme amplification factors of approximately 1.6-fold in mutant lysogens and 3.0-fold in parental lysogens (relative to the wild-type activities) are consistent with the fumA gene being normally located near the replication terminus (terC) and with the integration of λ fumA at the prophage attachment site (attI), because in growing cells a fumA gene at the latter site should be represented by more gene copies than a fumA gene near terC.

The fumarase activities of the λ fumB lysogens were not detectably different from the nonlysogenic strains (Table 2), and this is consistent with the failure of λ fumB to restore a

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Fumarase specific activitya</th>
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<tr>
<td></td>
<td></td>
<td>F → M</td>
</tr>
<tr>
<td>1535 fum</td>
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<td>0.2</td>
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<tr>
<td>1535(ANM540) fum (λ)</td>
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<tr>
<td>1535(AG134) fum (λ fumA+)</td>
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<td>4.3</td>
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<tr>
<td>1535(AG135) fum (λ fumA+)</td>
<td></td>
<td>3.4</td>
</tr>
<tr>
<td>1535(AG139) fum (λ fumB+)</td>
<td></td>
<td>0.2</td>
</tr>
<tr>
<td>1535(AG140) fum (λ fumB+)</td>
<td></td>
<td>0.2</td>
</tr>
<tr>
<td>NK-1 fum+</td>
<td></td>
<td>1.8</td>
</tr>
<tr>
<td>NK-1(ANM540) fum+ (λ)</td>
<td></td>
<td>2.6</td>
</tr>
<tr>
<td>NK-1(AG134) fum+ (λ fumA+)</td>
<td></td>
<td>5.5</td>
</tr>
<tr>
<td>NK-1(AG135) fum+ (λ fumA+)</td>
<td></td>
<td>8.5</td>
</tr>
<tr>
<td>NK-1(AG139) fum+ (λ fumB+)</td>
<td></td>
<td>2.8</td>
</tr>
<tr>
<td>NK-1(AG149) fum+ (λ fumB+)</td>
<td></td>
<td>3.3</td>
</tr>
<tr>
<td>1535(pGS54) fum fumA+</td>
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<td>129.0</td>
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<td>1535(pGS56) fum fumB+</td>
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<td>1.0</td>
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<tr>
<td>1535(pGS57) fum fumA+</td>
<td></td>
<td>31.7</td>
</tr>
<tr>
<td>1535(pGS58) fum</td>
<td></td>
<td>0.1</td>
</tr>
</tbody>
</table>

a Ultrasonic extracts of duplicate cultures grown in glucose minimal medium were assayed for the disappearance of fumarate (F → M) and conversion of malate to fumarate (M → F). The average specific activities are given as micromoles of fumarate transformed or produced per milligram of protein per hour.

Table 2. Fumarase activities of λ fum lysogens and plasmid-containing strains
Fum$^+$ nutritional phenotype to the mutant lysogens. One of several possible explanations for the weak transducing activity of the $\lambda$ fum$^B$ phages is that they encode some other hydratase which has a very low affinity for fumarate but can complement the fumarase lesion under the conditions of high amplification that obtain in phage-infected cells. Accordingly, the aconitase activities of the $\lambda$ fum$^A$ and $\lambda$ fum$^B$ lysogenic and nonlysogenic derivatives of strains 1535 and NK-1 were assayed. Specific activities of 0.12 to 0.24 $\mu$mol of aconitate transformed per mg of protein per h were obtained for different strains, but there was no correlation between aconitase activity and $\lambda$ fum$^B$ lysogeny. Thus, it was concluded that the $\lambda$ fum$^B$ transducing activity is not associated with aconitase, unless a phage promoter is essential for expressing the cloned gene. No other hydratases were tested.

Postinfection labeling of phage-cloned gene products. Phages containing the fum$^A$ and fum$^B$ regions inserted with opposite polarities were used in postinfection labeling studies to analyze the polypeptides expressed from the corresponding segments and to deduce their transcription polarities. Cultures were irradiated to reduce host gene expression before infection, labeling with $^{35}$S-methionine, and polypeptide analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. With a homimmune host, e.g., S159 (\textit{imm}$^{+19}$), gene expression is further limited to bacterial genes accompanied by their own promoters. Under these conditions, the $\lambda$ fum$^A$ phages (\textit{AG134} and \textit{AG135}) directed the synthesis of three polypeptides with $M_r$ of 61,500, 48,000, and 42,000 (Fig. 3a). With a $\lambda$-sensitive host (S159), cloned genes can also be expressed from the early or late phage promoter, $p_L$ or $p_R$, depending on the orientations of the genes relative to the phage genome. This orientation can be deduced by comparing the labeling patterns obtained during early and late phases of infection with phages having inserts of opposite polarities. The results for the $\lambda$ fum$^A$ phages indicate that the genes corresponding to the three polypeptides are transcribed with leftward polarity in \textit{AG134} (Fig. 3b and c). The degree of incorporation of label into the $M_r$ 42,000 product was relatively poor with the shorter early and late pulses, but the pattern was established by extending the period of autoradiography and by using 7.5% acrylamide gels (data not shown). An explanation for the poor incorporation emerged when the $M_r$ 42,000 polypeptide was identified as the product of the man$^A$ gene (see below), which presumably needs man-

![FIG. 3. Autoradiogram of $^{35}$S-labeled polypeptides synthesized in UV-irradiated hosts after infection with $\lambda$ fum transducing phages and fractionation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Labeling was (a) from 2 to min 32 after infection with a $\lambda$ imm$^{+1}$-immune host and (b) 4 to 12 min, early, or (c) 22 to 30 min, late, with a $\lambda$-sensitive host. Molecular weights of polypeptides ($\times 10^4$) are shown. The phages corresponding to individual tracks are: 1, uninfected bacteria; 2, lamNS40 (vector); 3, AG134 (\textit{fum}A); 4, AG135 (\textit{fum}B); 5, AG139 (\textit{fum}B); 6, AG140 (\textit{fum}B).]
nose to induce better expression and incorporation. Two additional labeled polypeptides of uncertain origin, M, 46,000 and 40,000, were detected in 7.5% gels with the λ-sensitive host, and the patterns of incorporation indicated a leftward transcription polarity in λG134.

In parallel studies, the λ fumB phages (AG139 and AG140) were found to direct the synthesis of two polypeptides, M, 61,500 and 33,000, in the immune host, and the labeling patterns obtained with the λ-sensitive host showed that the gene encoding the M, 61,500 product of λ fumB is transcribed with leftward polarity in λG140 (Fig. 3). The orientation-specific labeling of the M, 33,000 component was obscured by a phage protein in the λ-sensitive host. Several other specific polypeptides were detected when the phage promoters were active, and their sizes and transcription polarities were established with 7.5% gels (data not shown). The sizes of these polypeptides were estimated as M, 56,000, 52,000 and 43,000 (leftward in λG140). They could be products of fused phage plus bacterial genes, bacterial genes separated from their promoters during cloning, or poorly expressed bacterial genes that are transcribed more effectively when coupled to a phage promoter.

Restriction maps of the fumA and fumB segments of the bacterial DNA. Restriction sites in the HindIII fragments containing fumA and fumB were compiled by using DNA from phages λG134 (λ fumA), λG140 (λ fumB), and λNM540 (the vector and control) and by single and double digestion with different combinations of 10 restriction endonucleases. Restriction sites for three additional endonucleases (AvaI, ClaI, and PstI) were located with plasmids pGS54 (fumA) and pGS56 (fumB) (see below). The fumA fragment contained single targets for AvaI, EcoRI, PstI, and XhoI, but no cleavage sites for BamHI, BglII, Clal, KpnI, SalI, Smal, SphI, and SstI. The fumB fragment contained two targets for AvaI, BamHI, BglII, and PstI and single targets for EcoRI and SphI, but they contained no cleavage sites for Clal, Kpn, SalI, Smal, SstI, and XhoI. The coordinates (in kilobases) relative to the left-hand HindIII sites of both fragments are shown in Fig. 4.

Construction and characteristics of plasmid derivatives. The fumA and fumB fragments were transferred from λ fum phages to the HindIII site in the tet region of the plasmid vector, pBR322 (5). The aims were to develop the physical maps of the fum regions, to identify the fum and man gene products, and to assign each product to specific segments of the cloned DNA. Ligation mixtures containing HindIII digests of phages λG135 or λG140 and plasmid pBR322 were used to transform strains PM191 λ and 1535, the ampicillin-resistant (Amp') transformants being screened for tetracycline susceptibility (Tet') and Tet' Fum' products, respectively. Strains with plasmids pGS54 and pGS55 containing the fumA fragment (6.2 kb) inserted with different polarities were readily isolated. No Amp' Tet' products were obtained from λ fumB, but one Amp' Tet' derivative of strain 1535, detected by its weak growth on fumarate medium, contained a plasmid (pGS56) with the fumB fragment (8.2 kb) inserted in pBR322. The results suggest that in plasmid pGS56, the plasmid tet gene is expressed from a promoter in the fumB fragment. It would also appear that in a multicopy situation the fumarase defect of strain 1535 is complemented, albeit weakly, by the fumB fragment, just as it is in the lytic rather than lysogenic responses to λ fumB. The structures and characteristics of the plasmids pGS54, pGS55, and pGS56 and of plasmids derived from them are summarized in Fig. 5.

Deletion derivatives of plasmid pGS54 were obtained by religating the 7.8-kb (pGS57) and 5.8-kb (pGS61) fragments isolated from EcoRI and PstI digests and selecting for Amp', Fum+, or Man+ transformants of strains 1535 (fumAλ) and GMS407 (manA). Plasmid pGS57 retained both the fumA+ and manA+ functions, but pGS61 retained only manA+ (Fig. 5). This locates the manA gene in the 2.2-kb PstI-HindIII fragment and the fumA gene in the overlapping 3.5-kb EcoRI-HindIII fragment.

Deletion derivatives of plasmid pGS55 were sought by the same method, but only one type was recovered, pGS58 (Fig. 5). The fumA+ and manA+ functions had been deleted with the EcoRI fragment during the construction of this 7-kb plasmid, confirming the location of the corresponding genes. No transformants were recovered with the 7.6-kb PstI fragment of.

![Restriction maps of the fumA and fumB segments of the bacterial DNA.](http://jb.asm.org/Downloaded from http://jb.asm.org/)
pGS55, presumably because essential parts of all three selectable genes (fumA, manA, and amp) reside in the 3-kb PstI fragment that is deleted in construction (Fig. 5). This suggests that the PstI site in the fumA region is actually in the fumarase gene.

Two additional plasmids, pGS62 and pGS63, were constructed directly from mixtures of PstI digests of pGS55 and pBR322 by selecting Tet' Amp' transformants of strain PM191/λ. Plasmid pGS63 resembled PGs61 in expressing the manA gene, but it is more versatile because it has a functional tet gene (Fig. 5). The failure of pHS62 to transform the fumarase mutant confirmed the earlier suggestion that the fumarase gene spans the critical PstI site linking the 1.3- and 2.2-kb (manA) fragments (Fig. 5).

**Enzymology of plasmid-containing strains.** The fumarase specific activities of four derivatives of strain 1335 containing plasmids pGS54, pGS56, pGS57, and pGS58 are listed in Table 2. The results are consistent with the Fum+ nutritional phenotypes conferred by pGS54 and pGS57 and the weak Fum+ phenotype of the pGS56-containing strain. They also confirm the location of the fumarase gene (fumA) in the 3.5-kb EcoRI-HindIII segment of cloned DNA. Fumarase was amplified approximately 50-fold and 12-fold with the fumA plasmids pGS54 and pGS57, respectively, and approximately 40% of wild-type activity was detected with the fumB plasmid. The difference between the highly amplified strains may stem from the presence of ampicillin in the inocula used with pGS54 (and pGS56) but not with pGS57 (and pGS58): the ampicillin may have limited plasmid elimination in the pGS54 culture.

**Identification of the fumA and manA gene products.** The maxicell procedure was used to detect the proteins expressed from different plasmids and to correlate these products with specific functions and DNA fragments (Fig. 6). Three polypeptides were specifically expressed from the fumA region (6.2-kb fragment) of plasmid pGS54; their molecular weights were 61,500, 48,000, and 42,000. By comparing the labeling patterns, structures, and properties of the pGS54 derivatives, it was deduced that the M, 42,000 component, the polypeptide expressed from all the manA plasmids, is mannosolphosphate isomerase. Likewise, the M, 48,000 component expressed from plasmid pGS57 (manA fumA) but not from pGS63 (manA+), corresponds to fumarase, the fumA gene product. The M, 48,000 component appears to be the product of an unidentified gene (designated g48) that contains the EcoRI target in the fumA region (Fig. 5), because this polypeptide was not expressed from either of the adjacent fragments that are cloned in plasmids pGS57 and pGS58. No polypeptides specifically expressed from plasmid pGS58 could be detected. The polypeptides specifically expressed from the fumB region (8.2-kb fragment) of pGS56 were of M,

**FIG. 5.** Physical maps of plasmids containing segments of the fumA and fumB regions of bacterial DNA transferred λ fum transducing phages to plasmid pBR322. Bacterial DNA is denoted by open bars and vector DNA by single lines. The scale drawings show linear representations of the circular plasmid molecules with targets for endonucleases as follows: H, HindIII; R, EcoRI; P, PstI; B, BamHI. A scale (each division = 1 kb) and the sizes of three key fragments are shown. The phenotypic characteristics conferred by the plasmids are as follows: F', Fum'; M', Man'; A', Amp'; T', Tet'.

**FIG. 6.** Autoradiogram of 35S-labeled polypeptides expressed from cloned genes. Plasmid-containing cultures were labeled for 2 h by the maxicell procedure in two separate experiments. Molecular weights of polypeptides (×10^3) are shown. Individual tracks represent: 1, pGS54; 2, pGS55; 3, pGS57; 4, pGS58; 5, pGS62; 6, pGS63; 7, pBR322.
By nutritional selection, two phenotypically and physically distinct classes of fumarase-transducing phages (λ fumA and λ fumB) were isolated from recombinant phage pools. The strongly transducing class (λ fumA) appeared to encode the major fumarase and its promoter because the lesion of a fumarase-negative mutant was complemented by a λ fumA prophage. In conjugation studies, the fumarase gene (fumA) has been located at 35 to 36 min, near the terminus of chromosome replication (terC) and very close to the mannosephosphate isomerase gene (manA). The manA gene was also transduced and complemented by the λ fumA transducing phages. By a combination of restriction analysis, subcloning, complementation, and gene product labeling, the 6.2-kb fragment cloned in the λ fumA manA phages was shown to encode at least three genes. Their relative positions, sizes, and transcription polarities are summarized in Fig. 7. The basic fumarase subunit was identified as the polypeptide of apparent molecular weight 61,500, whereas mannosephosphate isomerase corresponds to the 42,000 component. Together, these enzymes require almost all the coding capacity of the 3.5-kb EcoRI-HindIII segment of the cloned DNA. The third gene (g48), identified only by its gene product (Mr, 48,000), appeared to be expressed from within the same 3.5-kb fragment; it is not known whether it forms part of a fum operon. Initially the Mr, 48,000 component was regarded as the most likely candidate for fumarase by size comparability with mammalian fumarases, Mr, 48,500 (2), and preliminary evidence for the Bacillus subtilis enzyme. A series of fumarase-transducing phages (λ citG) containing overlapping fragments of B. subtilis DNA has been isolated, and the only common product detected in postinfection labeling studies was a Mr, 49,000 polypeptide presumed to be fumarase (A. Moir, J. Gen. Microbiol., in press; J. R. Guest, unpublished observations). However, it is now clear that the E. coli enzyme is larger than the mammalian and possibly the B. subtilis enzymes. Size differences between some tricarboxylic acid cycle enzymes from gram-negative and gram-positive organisms have been noted, and the latter tend to bear closer resemblance to their mammalian equivalents (27).

Recent studies by Bouché and co-workers (6, 7) on the structure of the terC region confirm the location of the fumA gene and define the orientation of the fumA fragment relative to the genetic linkage map (see Fig. 7). The HindIII restriction fragment containing the fumA gene is readily identifiable in the physical map deduced from terminal labeling studies by Bouché (6). The fragment also corresponds very strikingly to the physical map based on DNA hybridization studies with a man::Mu insertion strain (7). By reference to the Bouché map it can also be concluded that the two HindIII fragments of 5.4 and 6.2 kb (fumA) cloned in λG138 (Fig. 2) are not adjacent in the bacterial chromosome. Presumably the λG138 insert arose by recombination rather than partial digestion. In fact, the critical segment of the bacterial chromosome has recently been isolated by Blanco et al. (4) in a ColE1-uid hybrid plasmid from the Clarke and Carbon gene bank. This plasmid (pU1) overlaps the fumA fragment at the manA end. The location of an intact manA gene in the Psrl-HindIII fragment was established.

The fumarase activities of λ fumA lysogens were consistent with a location for fumA near the terminus of chromosome replication and with fumA gene expression from the fumarase promoter in a gene dosage-related manner. Cloning in multicopy vectors resulted in high fumarase activities, and strains containing the fumA plasmids pGS34 and pGS57 should provide highly enriched sources of fumarase for enzymological and protein studies. The organisms could also be of use in the industrial production of L-malic acid, which involves bacteria having high fumarase activities (25).

The nature of the weak transducing activity of the λ fumB phages was not defined, but there are
several possible explanations. The simplest is that *E. coli* possesses a gene (*fumB*) encoding a second fumarase (with weak or poorly expressed activity) or some other hydratase which has a low affinity for fumarate. The fumarase activity reached only 40% of the parental level with the multicity plasmids, but this at least suggests that the weak activity and poor complementation may not be due to separation of the *fumB* gene from its promoter. It is interesting but possibly coincidental that the *fumB* fragment expresses a polypeptide which has the same size as the *fumA* gene product. An alternative explanation is that *fumB* encodes a CR63-derived translational suppressor of the *fumA1* mutation. These and other possibilities are currently under investigation.

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LITERATURE CITED


